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Formation of DNA Adducts in 10T1/2 Mouse Embryo Fibroblasts Incubated with Benzo(a)pyrene or Dihydrodiol Oxide Derivatives

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Formation of DNA Adducts in 10T½ Mouse Embryo Fibroblasts Incubated with Benzo(*a*)pyrene or Dihydrodiol Oxide Derivatives[1]

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ABSTRACT

Binding of benzo(*a*)pyrene (BP) metabolites to DNA was studied in the mouse embryo fibroblast 10T½ cell line. The cells metabolized BP to its diastereoisomeric 7,8-dihydrodiol-9,10-oxides, (±)-7β, 8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene and (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene, which formed covalent adducts with cellular DNA. Detailed analysis by high-pressure liquid chromatography indicated that a deoxyguanosine adduct, N^2-(10*S*-[7*R*,8*S*,9*R*-trihydroxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene]yldeoxyguaninosine, was the predominant DNA adduct formed in cells exposed to BP. This adduct is also the major one found in human tissues exposed to BP. Exposure of cells to (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene and (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene, rather than to BP, produced several additional adducts on DNA. During a 68-hr posttreatment incubation, about 50 to 70% of the adducts were removed from the cellular DNA. No major differences between the relative rates of excision from cellular DNA of the individual (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene and (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene adducts were found. These results provide evidence that when 10T½ cells are exposed to BP there is stereoselective synthesis and covalent binding of BPDE mainly to the N^2 group of deoxyguanosine. This similarity with results obtained in human tissues makes the 10T½ cell an attractive model system for studying the action of BP and related polycyclic hydrocarbons.

INTRODUCTION

BP[3] and related polycyclic aromatic hydrocarbons are abundant in the human industrial environment, and such pollutants have been linked to human cancer (8, 10). Metabolic activation of BP by mixed-function oxidase in conjunction with epoxide hydrase results in covalent binding to cellular proteins and nucleic acids in the intact animal and in tissue culture (32). Recent studies have suggested that the major reactive *in vivo* intermediate of BP is 7,8-dihydrodiol 9,10-oxide (9, 29). Two isomers, BPDE I and BPDE II, have been identified and linked directly to mutagenicity (9, 25, 31, 33, 34) and carcinogenicity (17, 19, 21). Each BPDE isomer can exist in 2 enantiomeric forms (15), and each of these can bind covalently, via either *cis* or *trans* opening of the epoxide ring, to one of the many nucleophilic sites on DNA. Thus, the number of all possible adducts is large. In the *in vivo* systems which have been studied, however, the number of BP-DNA adducts found was limited, although variable from one system to another. In human and bovine bronchial explants, essentially only one product, 10-*trans*-7R-BPDE I-dGuo, was formed (13, 14, 24). The 7S-enantiomer of BPDE I-dGuo and the BPDE II-dGuo adducts were detected in DNA from mouse skin exposed to BP (18). Comparable amounts of BPDE I-dGuo and BPDE II-dGuo were formed in human alveolar tumor cells (6) and in baby hamster kidney cells (28), whereas BPDE I-dGuo was the predominant one of the 2 products in BP-treated secondary mouse embryo fibroblasts (28) and in explants from human lung or colon (1, 27). In primary hamster embryo cells, at least 3 dGuo products and a small amount of deoxycytidine and deoxyadenosine adducts were found (11). The above findings point toward variable specificity in metabolism and reactions of BP metabolites with DNA *in vivo*.

Two processes are expected to contribute to the steady state level of BP adducts on cellular DNA, namely, binding of its metabolites to DNA and their rates of excision. Removal of BPDE adducts from DNA has been shown to occur in several systems. There is evidence that not all adducts are removed from DNA at equal rates (6, 11, 28). Assuming that metabolism, binding to DNA, and removal of the carcinogen occur simultaneously in a cell exposed to BP, it is difficult to determine which of the processes, or their combination, is responsible for the differential DNA modification in various systems (3). One way of simplifying the system is elimination of the metabolic activation step by treating the cells with preformed BPDE (6). Another is treatment of cells with BP for only a short period of time and then monitoring the repair in the absence of the carcinogen. The shortcoming of the latter method is the low level of binding obtained and the possibility of continued binding to DNA of residual BP and its metabolites during the repair phase of the study.

In the present study, we have analyzed the extent of DNA binding and the types of adducts formed when confluent cultures of the mouse embryo fibroblast cell line 10T½ are exposed to radioactive BP or BPDE. This cell line is of particular interest because it is highly sensitive to postconfluent inhibition of cell division (23) and is quite susceptible to transformation by BP and related polycyclic aromatic compounds (4, 16, 22). It is also 100-fold more sensitive to transformation by BPDE than by BP (20, 30). This mammalian system provides an

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[3] The abbreviations used are: BP, benzo(*a*)pyrene; BPDE I, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; BPDE II, (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; BPDE, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; 10-*trans*-7R-BPDE I-dGuo, N^2-(10*S*-[7*R*,8*S*,9*R*-trihydroxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene]yl-deoxyguanosine; dGuo, deoxyguanosine; HPLC, high-pressure liquid chromatography.

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interesting contrast to another short-term assay, the Ames bacterial mutagenesis system.

MATERIALS AND METHODS

Chemicals. [^{3}H]BP (specific activity 40 mCi/μmol) was purchased from Amersham Searle Corporation. [^{3}H]BPDE I (specific activity, 223 μCi/μmol) and [^{14}C]BPDE II (specific activity, 29.6 μCi/μmol) were obtained from Dr. J. Engel, Midwest Research Institute, Kansas City, Mo., and Dr. J. Keith, IIT Research Institute, Chicago, Ill., through the Chemistry Operational Unit, National Cancer Institute. All enzymes were from Worthington Biochemicals, Freehold, N. J. Polydeoxyguanylic acid, polydeoxyadenylic acid, and polydeoxycytidylic acid were from P.L. Biochemicals, Milwaukee, Wis.

Cell Culture. The C3H10T½ clone 8 (26) cell line was kindly provided by Dr. John Bertram, Roswell Park Memorial Institute, Buffalo, N. Y. 10T½ cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were grown at 37° in 5% CO_2–air and 95% humidity. They were passaged every 10 days by dissociation, using 0.1% trypsin-Versene and plating at 5×10^4 cells/6-cm tissue culture dish. At confluency, there were 10^6 cells/plate.

Treatment of Cells with BP and BPDE. Confluent cultures were treated with the carcinogens within 24 hr after reaching confluence. [^{3}H]BP (final specific activity, 4 mCi/μmol) was added to each 6-cm culture plate. In the studies with labeled BPDE, the growth medium was removed and 4 ml of serum-free medium were added per plate, then 10 μl of labeled BPDE in dimethyl sulfoxide were added with rapid mixing. The final concentrations were [^{3}H]BPDE I (0.3 μg/ml) + [^{14}C]BPDE II (1.2 μg/ml). The cells were incubated in the dark for 12 or 24 hr with [^{3}H]BP or for 2 hr with labeled BPDE. Exposure to the carcinogens was terminated by removal of the radioactive medium. In the posttreatment incubation experiments, cells were washed 4 times with fresh serum containing medium (lacking carcinogen) and incubated in the dark for an additional 68 hr. At the end of each experiment the medium was removed, and cells were washed once with phosphate-buffered saline containing 70 mM NaCl, 3.3 mM KCl, 10.7 mM $Na_2HPO_4 \cdot 7H_2O$, and 1.44 mM KH_2PO_4 and collected by scraping the cells from the plates.

Nucleic Acid Extraction and Purification. DNA from the pelleted Triton X-100-treated nuclei (2) was solubilized in 1% sodium dodecyl sulfate solution, pH 6.9, containing 15 mM NaCl and 1.5 mM citrate. It was then extracted 5 to 6 times with water-saturated phenol containing 0.1% 8-hydroxyquinoline and 0.1% sodium dodecyl sulfate and precipitated twice by adding a 2-fold volume of ethanol in the presence of sodium acetate (0.2 mmol/ml), pH 5.0. RNA, from intact cells treated with Triton X-100, was extracted, precipitated, and purified as previously described (2). The specific activity of the modified DNA was determined as described (11) and expressed as mol of BP residues per 10^6 mol of nucleoside residues.

***In Vitro* Modification of Deoxyribopolymers and Column Chromatography.** Five A_{260} units of nucleic acid in 2 ml of 10 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$, pH 7.9, were incubated at 37° consecutively with 100 units of DNase I and 50 units of micrococcal nuclease (4 hr), 10 units of alkaline phosphatase (2 hr), 0.2 unit of spleen phosphodiesterase (18 hr), 0.2 unit of venom phosphodiesterase (4 hr), and again with 10 units of alkaline phosphatase. Under these conditions, 90 to 95% of the radioactivity was released as modified deoxyribonucleosides. These were separated from unmodified deoxyribonucleosides on Sephadex LH-20 by elution with methanol (15). Modified deoxyribonucleosides thus obtained were analyzed by HPLC as described previously (12).

RESULTS

Binding of BP and BP Dihydrodiol Oxide to DNA in Confluent 10T½ Cells. 10T½ cells were grown until confluent, and within 24 hr after reaching confluency they were exposed to the carcinogen. In one experiment, [^{3}H]BP treatment was for 12 hr, and in another, it was for 24 hr. Because the half-lives of BPDE I and II are short (19, 35), the BPDE treatment was limited to 2 hr. At the end of these exposure times, the medium was removed. The cells assigned for a posttreatment incubation were carefully washed 4 times with fresh medium containing serum but no carcinogen. No radioactivity was detected in the medium of BPDE-treated and washed cells. [^{3}H]BP and/or its metabolites were more difficult to remove, and there was radioactive material still present after washing. To avoid possible damage to cells and other artifacts, washing was not continued. One would expect, therefore, some additional binding of [^{3}H]BP metabolites during the posttreatment incubation. The cells assigned for collection were washed once with phosphate-buffered saline and scraped off the plates, and the DNA was extracted.

The extent of modification of DNA at various time points and under the different labeling conditions is shown in Table 1. After 12 hr of treatment with [^{3}H]BP, only a small amount of DNA adducts (2.7 BP residues per 10^6 nucleotides) was found. Increasing the [^{3}H]BP treatment from 12 to 24 hr resulted in about a 10-fold increase of binding to DNA. The additional 68 hr of incubation in fresh medium lacking [^{3}H]BP resulted in about a 40% decrease in the specific activity of the DNA. This decrease appears to be due to partial excision of the BP adducts via a DNA repair process. It cannot be attributed to an approximate doubling of the DNA, without further labeling of the newly synthesized DNA, because in these confluent 10T½ cells there is negligible DNA replication (30). Nor is it due to cytotoxicity and cell replacement since the concentrations of [^{3}H]BP and labeled BPDE used in the present study are not cytotoxic (30). Parallel studies (not shown here) with cultures prelabeled with [^{3}H]BP indicated that during the posttreatment incubation, although there was a decrease in the specific activity of the DNA, there was not a decrease in specific activity of the RNA. This is also consistent with DNA excision repair since, if the reduction in DNA specific activity during the posttreatment incubation was simply due to cell proliferation, then one would expect a parallel decrease in the specific activity of RNA. Previous studies in primary hamster embryo (11) or human lung (7) cultures have also provided evidence for DNA excision of BP-DNA adducts.

When cells were exposed to a mixture of [^{3}H]BPDE I and [^{14}C]BPDE II for 2 hr, the extents of modification of DNA were about 4-fold greater than the modification obtained after a 24-hr incubation with [^{3}H]BP (Table 1). During the 68-hr posttreatment incubation, there was, in both cases, a significant reduction in the specific radioactivity of the DNA. Since BPDE I and

Table 1

Binding to DNA in cultures of 10T½ cells exposed to radioactive BP or BPDE

For additional details, see "Materials and Methods."

Compound added	Duration of treatment (hr)	Posttreatment incubation (hr)	Extent of DNA modification[c]	Relative abundance of individual peaks (%)					
				Peak[a] 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
[^{3}H]BP	12	0	2.7	7	ND[b]	7	59	27	ND
	24	0	30	ND	ND	7	83	10	ND
	24	68	20	ND	ND	5	85	10	ND
[^{3}H]BPDE I + [^{14}C]BPDE II	2	0	130	10	23	10	19	23	15
	2	68	38	8	36	7	18	18	13

[a] See Charts 1 and 2.
[b] ND, compound was not detected.
[c] BP residues/10^8 deoxynucleoside residues.

BPDE II have half-lives in aqueous solution of less than a few min (35), the labeling in these studies can be considered as a pulse exposure. A decrease in the specific activity of the DNA during the posttreatment incubation better reflects the excision repair process than the studies with [^{3}H]BP-treated cells, where both repair and continued binding occur during the posttreatment incubation.

To determine the types of DNA adducts formed and to examine the rates of removal of individual adducts, the above DNA samples were digested enzymatically, and the profiles of the deoxyribonucleoside adducts were examined by HPLC.

Analysis of BP-Deoxyribonucleoside Adducts by HPLC. The HPLC profiles obtained after enzymatic hydrolysis and Sephadex LH-20 column chromatography of DNA from the [^{3}H]BP-treated cells are shown in Chart 1. Identification of peaks was made by comparison of the elution volumes with those of markers obtained by modifying polydeoxyguanylic acid, polydeoxyadenylic acid and polydeoxycytidylic acid with BPDE I or BPDE II as described in "Materials and Methods." The relative abundances of each modified nucleoside, expressed as percentage of the total radioactivity recovered from the HPLC column, are given in Table 1. Four major components, designated BP-DNA-1, -3, -4, and -5, were present in DNA from cells exposed for 12 hr to [^{3}H]BP (Chart 1*A*). Their elution volumes coincided with those of BPDE-dCyd, the 7S enantiomer of BPDE-dGuo, 10 *trans*-7R-BPDE I-dGuo, and BPDE II-dGuo, respectively. At 24 hr (Chart 1*B*), BP-DNA-1 could not be detected. On the other hand, the absolute amounts of the compounds BP-DNA-3, -4, and -5 increased between 12 and 24 hr. During this interval, the relative content of BP-DNA 3 (6 to 7%) remained unchanged, the relative content of BP-DNA-4 increased from 58 to 82%, and the relative content of BP-DNA 5 decreased from 26 to 10% of the total radioactivity eluted. During the subsequent 68-hr posttreatment period, there was no appreciable change in the relative content of the compounds BP-DNA-3, -4, and -5 (Chart 1*C*; Table 1). It is of interest that at all time points the most prominent peak, Peak 4, was the compound which is most prominent in human bronchial explants exposed to BP (14).

Comparisons between Chart 1*C* and 1*B* may not provide direct information on rates of excision of the individual DNA adducts during the posttreatment incubation period. Changes in relative abundance of individual adducts could reflect their continued synthesis (due to the persistence of some [^{3}H]BP) as well as their rates of excision from DNA. For this reason, we treated the cells with the ultimate metabolites, BPDE I and BPDE II, and examined the resulting HPLC profiles as a function of time. This provided a more direct analysis of excision repair since, as discussed above, the short half-lives of BPDE I and II result in a pulse type of exposure.

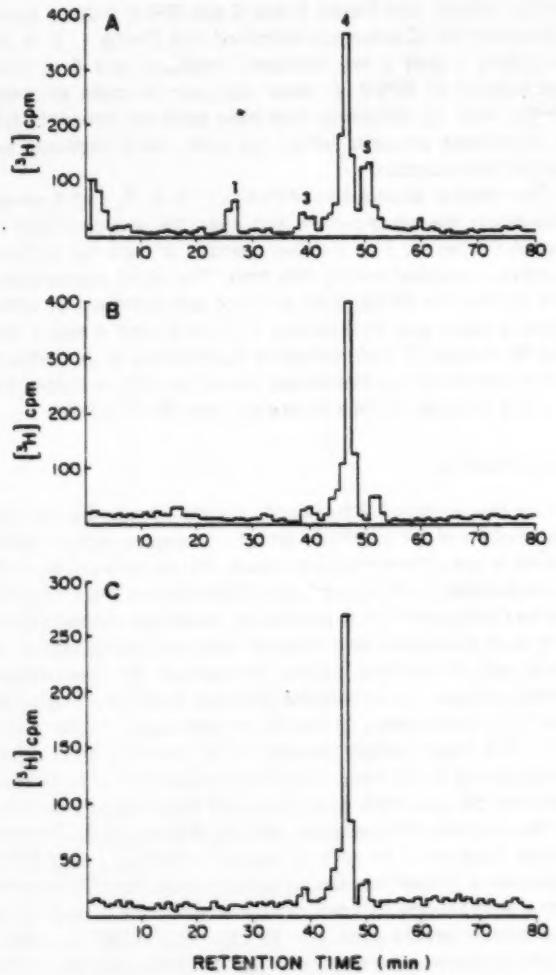

Chart 1. Profiles of DNA adducts formed in confluent 10T½ cultures incubated with [^{3}H]BP. *A*, DNA from 10T½ cells after 12 hr of exposure to [^{3}H]BP; *B*, DNA from 10T½ cells after 24 hr of exposure to [^{3}H]BP; C, DNA from 10T½ after 24 hr of exposure to [^{3}H]BP and 68 hr of posttreatment incubation.

Analysis of BPDE-Deoxyribonucleoside Adducts by HPLC. The HPLC profiles of DNA from 10T½ cells treated with [^{3}H]-BPDE I plus [^{14}C]BPDE II are shown in Chart 2. The DNA adducts are much more heterogeneous in cells exposed to labeled BPDE I plus BPDE II than in cells exposed to [^{3}H]BP (compare Charts 1 and 2). The level of binding of [^{14}C]BPDE II is slightly higher than that of [^{3}H]BPDE, but this may simply reflect its higher concentration in the medium. Six major peaks, designated 1 to 6, and at least 4 minor ones can be identified in Chart 2*A*. Identification of the individual adducts is based on comparisons of their retention times with those of markers, and in several cases their absolute stereochemistry is not known. The following assignments are, therefore, tentative. Peak 1 is BPDE I-dCyd, Peak 2 is BPDE II-dGuo, Peak 3 is the 7S enantiomer of BPDE I-dGuo, Peak 4 is the 7R enantiomer of BPDE I-dGuo, and Peaks 5 and 6 are BPDE II-dGuo adducts. Additional HPLC analyses indicated that Peaks 1, 3, 4, and 5 in Charts 1 and 2 are identical. Peaks 2 and 6 in Chart 2 (derivatives of BPDE II) were not seen in cells exposed to [^{3}H]BP (Fig. 1), indicating that they were not present on DNA in significant amounts when the cells were exposed to the parent hydrocarbon.

The relative abundance of Peaks 1, 3, 4, 5, and 6 remained essentially the same before and after the posttreatment incubation (Table 1). The relative amount of material in Peak 2, however, doubled during this time. The likely explanation for this is that the BPDE-DNA adducts are removed at different rates; a rapid one for Adducts 1, 3, 4, 5, and 6 and a slower one for Adduct 2. The biological significance of this difference is not clear since, as mentioned above, we did not detect Peaks 2 and 6 material in cells incubated with BP (Chart 1).

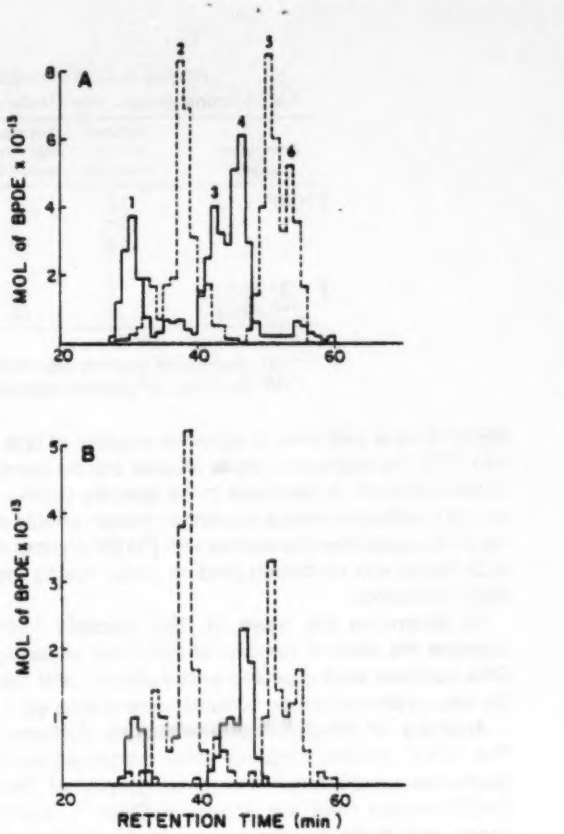

Chart 2. HPLC profiles of DNA adducts formed in confluent 10T½ cultures incubated with [^{3}H]BPDE I plus [^{14}C]BPDE II. *A*, DNA from 10T½ cells after 2 hr of exposure to diol-epoxides; *B*, DNA from 10T½ cells after 2 hr of exposure to diol-epoxides and 68 hr of posttreatment incubation; ——, BP I-DNA adducts; - - - -, BP II-DNA adducts. Different amounts of material were applied to the column in *A* and *B*; therefore, the mol of BPDE-DNA adducts per fraction bears no relationship to the extent of modification of DNA.

DISCUSSION

Our results indicate that confluent 10T½ cells are capable of metabolism of BP to BPDE I and, to a lesser extent, to BPDE II, which, in turn, form covalent bonds with base residues in DNA. Comparisons of HPLC profiles of DNA digests from the [^{3}H]BP-treated cells with HPLC profiles of model compounds indicate that both guanosine and cytidine residues participate in reactions with dihydrodiol oxides, presumably on their exocyclic amino groups. No adenosine adducts have been detected in the 10T½ cells, although they have been seen in other systems (11). The major adduct present in BP-DNA (Chart 1, *Peak 4*) corresponds to 10-*trans*-7R-BPDE I-dGuo (14) and represents between 59 and 85% of all modified nucleosides, depending on the duration of treatment with the hydrocarbon. Treatment of the cells for 2 hr with a mixture of BPDE I and BPDE II produces a greater number of adducts than does BP treatment, with a significantly different relative abundance of each adduct. The simpler profile seen with BP can, therefore, be attributed mainly to stereoselective synthesis of BPDE isomers by these cells, with a predominance of BPDE I. This interpretation is consistent with the findings of Koreeda *et al.* (18) in mouse skin and the findings by Yang *et al.* (36) in liver microsomes that there is stereoselective synthesis of BPDE I. In addition, the latter studies indicated preferential formation of specific enantiomers of BPDE I and BPDE II.

It is of considerable interest that at all time points examined in the present study, in 10T½ cells exposed to [^{3}H]BP the major adduct, the 7R enantiomer of BPDE I-dGuo, is the same as the major adduct found in DNA when human bronchial or colon explants were exposed for 24 hr to [^{3}H]BP (1, 13, 14). This similarity with human tissues makes the 10T½ cell an especially attractive model system for studies on BP-DNA interactions. The major adduct in 10T½ DNA is also the major DNA product found in mouse skin DNA and a human lung cell culture following exposure to BP (18, 27). There are considerable differences between these various systems, however, in terms of the relative abundance of other BP-DNA adducts. In hamster embryo cell cultures exposed to BP, for example, the profile of DNA adducts is quite complex, and there is a greater abundance of BPDE II adducts and deoxyadenosine adducts than in 10T½ cells (3, 11).

Our results also demonstrate that 10T½ cells are capable of excising the BP-DNA adducts. It has been suggested that in some systems BPDE II adducts are removed more rapidly from DNA than BPDE I adducts (6, 28) and that this lower rate of repair may be related to the extraordinary mutagenicity of BPDE I (5, 6). Our preliminary results suggest that this is not the case with 10T½ cells, although additional studies on the question of differential rates of excision of the multiple adducts are required.

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